THE BACTERIOLOGICAL AND CHEMICAL EVIDENCE OF THE OCCURRENCE OF A HEXOSE SUGAR IN NORMAL MILK*

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One of the noteworthy cultural reactions of the organisms of the typhoid-paratyphoid-dysentery group is the production of a lilac color, either transient or permanent, in litmus milk. Theobald Smith[1] appears to have been the first to have called attention to this phenomenon. He pointed out that many organisms which ferment dextrose but not lactose produce this color reaction, and that various cultures of these bacteria made from the same milk exhibit the same degree of color change, practically never more nor less for one variety of organism than another, suggesting strongly that each variety is acting on the same substance in the milk, probably a second carbohydrate.

The purpose of this investigation was to determine by bacteriological and chemical examination the substance or substances in milk to which this reaction is attributable.

BACTERIOLOGICAL EVIDENCE

Decomposition Products of the Various Milk Constituents as Possible Sources of Acid Formation.—Milk consists essentially of proteins, fats, salts and carbohydrates, dissolved in water or in colloidal solution.

Proteins may be tentatively ruled out as a source of this reaction by the fact that the products of microbic action on them are usually basic rather than acid in character. A number of organisms of which *B. alkaligenes* is an example, which do not ferment carbohydrates, produce a progressively alkaline reaction in this medium as a result of their action on the milk proteins.

While fats cannot be ruled out by *a priori* considerations, nevertheless the organisms mentioned above do not appear to decompose fats as readily as they decompose proteins or utilizable carbohydrates; moreover, the color-change in litmus milk cultures of typhoid bacilli,

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1. *Jour. Boston Soc. Med. Sc.*, 1897, 2, p. 236.

for example, is found to be the same, whether it is observed in skimmed milk (0.2 per cent. fat) or whole milk (3.5 per cent. fat). If the reaction in this specific instance depended on the utilization of fats, the difference in the fat content of the milk in the instance cited should cause a noticeable difference in the intensity of the color-change.

The salts of milk appear to take no prominent part in their reaction, since an increase in hydrogen ion can occur only by removal of the metal ions from solution; and inasmuch as this color-change may be observed also in dextrose (0.1 per cent.) broth cultures whose salt content is known, an explanation of the phenomenon on the basis of the removal of metal ions of the salts in broth is obviously ruled out.

The tentative exclusion of three of the possible sources of this acid formation focuses attention on the carbohydrate content of milk. With respect to the carbohydrates in milk there is diversity of opinion: some observers claim that lactose alone is found normally in it; others admit that other carbohydrates may occur as normal constituents. Most investigators, however, are non-committal on this point.

The regularity with which this faint acidity is produced in litmus milk by those organisms which are known to ferment dextrose but not lactose would suggest, as pointed out by Theobald Smith, that the color-change is due to small amounts of acid produced by the fermentation of some hexose present normally in milk in small amounts. If this hexose is derived from lactose by hydrolysis, some plausible explanation for the limitation of the cleavage of lactose must be produced. On the other hand, if the color change is due to the fermentation of a portion of the lactose, then one must explain why these organisms form so little acid in the presence of so much lactose; their failure to use more of the lactose cannot be due to any inhibiting action of the acid, since these organisms are able to increase the acid reaction of the culture to the point of precipitating the casein, if dextrose be added to the litmus milk in amounts of from 0.5 to 1 per cent.

It has been shown by Kendall and others[2] that utilizable carbohydrates exhibit a sparing action for proteins; in other words, when these two food elements are present in a culture the organisms, if able to utilize the carbohydrate at all, will use the latter in preference to the protein, at least for their energy requirement. The sequence of color changes observed in litmus-milk cultures of these organisms is readily

2. *Jour. Med. Research,* 1911, 25, p. 117; *Jour. Biol. Chem.,* 1912, 11, p. 13; *Jour. Am. Chem. Soc.,* 1913, 30, p. 4.

explainable on the basis of this protein-sparing action of utilizable carbohydrates. If some dextrose is present, the organisms mentioned above do not ferment lactose. For example, *B. paratyphosus beta, B. enteritidis,* the bacillus of hog cholera, and the Flexner and Shiga types of the dysentery bacillus, as stated previously, produce this lilac color in litmus milk, but after two to eight days, depending on the organism used, the cultures change from this lilac color, "initial acidity," to a blue color, "permanent alkalinity." This phenomenon is explained readily by assuming that a small amount of hexose present at the start is soon exhausted, and the organisms are then forced to act on the proteins for their energy requirement. In the course of the protein decomposition basic substances are formed and the alkaline reaction follows. On the other hand, *B. typhosus* and *B. paratyphosus alpha,* which also produce this initial lilac color change, do not eventually exhibit an alkaline reaction, that is, being dextrose-fermenting organisms, they ferment the hexose of the milk, but their subsequent action on the proteins of the milk does not result in the formation of appreciable amounts of alkaline products. They can, however, give first an acid and then an alkaline reaction in litmus milk if sugar-free beef broth be added to it, or, as shown by Theobald Smith, if 0.1 per cent. dextrose bouillon colored with litmus be used instead of litmus milk. Theobald Smith suggests that this is probably due to the fact that these organisms attack the proteins of the beef broth readily, but do not decompose milk proteins readily. If these organisms act at all on milk proteins, the products are not appreciably alkaline, as is shown by the permanency of the acid reaction which appears during the first twenty-four hours of incubation in litmus milk.

POSSIBLE ORIGIN OF A HEXOSE SUGAR IN STERILE MILK

There are at least two explanations which might account for the presence of hexose in milk. Either it is present as (*a*) dextrose and galactose, the products of hydrolytic cleavage of lactose, produced by the high temperature and pressure incidental to the process of sterilization in the autoclave, or as (*b*) dextrose which has passed from the circulating blood of the cow into the milk during the normal process of lactation.[3]

(*a*) With regard to the first possibility, it should be noted that litmus milk is prepared by adding litmus solution to fresh milk and

3. Seegan: *Pflüger's Arch.,* 1887, 50, p. 48; Miura: *Ztschr. f. Biol.,* 1895, 32, p. 279.

sterilizing it at 120 C. for fifteen minutes. Lactose is said to be hydrolyzed readily at high temperatures, its hydrolytic products being dextrose and galactose. This hydrolysis would explain the presence of traces of hexose sugar in milk, if it occurred regularly during the process of sterilization.

(*b*) On the other hand, a consideration of the anatomy of the mammary gland suggests that this sugar may be simply dextrose, and that it may be secreted as a normal constituent of milk. The mammary gland is richly supplied with blood-vessels and lymphatics which lie in intimate contact with the cells actively engaged in the production of milk. During lactation the secretory cells increase in length, extending toward the lumen of the gland tubules; finally, the much-distended cells rupture, and the fluid contained in them escapes at the lumen end of the gland into the alveolus. It is fair to assume, therefore, that in a site of such physiological activity, involving a rich blood-supply, some of the dextrose of the blood might appear regularly in the secretion. Various other body fluids, such as normal urine (Baisch[4]), the transudates and exudates, and even the vitreous humor (Pantz[5]) have been shown to contain dextrose (although in much smaller amounts), and its presence is explained by a similar assumption, that is, that this dextrose comes from the circulating blood of the animal.

BACTERIOLOGICAL TESTS TO DETERMINE THE ORIGIN OF THE HEXOSE SUGAR IN STERILE MILK

The following experiments were made to determine if the lactose of neutral milk is hydrolyzed to dextrose and galactose when heated in the autoclave for fifteen minutes at a temperature of 120 C.

One hundred c.c. of milk were inoculated with *B. paratyphosus alpha* and incubated forty-eight hours. This culture, when it was colored with litmus, showed the lilac reaction characteristic of cultures of organisms fermenting sugars with six carbon atoms. This lilac-colored sample was cautiously neutralized with N/10 NaOH until the color matched that of a sterile litmus milk sample used as a color control.

In the tests requiring color comparisons proportionate amounts of milk and litmus were always used, since cultures containing different proportions of litmus show varying intensities of color changes. One may obtain milk free from the fermentable substance, but identical in color with sterile unchanged litmus milk, by this procedure.

The sample was now divided into two parts, D + and D°, and 0.05 per. cent. dextrose added to part D +. Both portions were then autoclaved at 120 C.

4. *Ztschr. f. Physiol. Chem.*, 1895, 20, p. 249; also Lemaire: *Ibid.*, 1895, 21, p. 442.
5. *Ztschr. f. Biol.*, 1895, 32, p. 236.

for fifteen minutes. It is evident that if autoclaving causes appreciable hydrolysis of lactose in neutral milk, both D + and D° will now contain hexose sugars, dextrose and galactose, whereas if hydrolysis has not taken place only D + will contain a hexose sugar, that is, dextrose, which, it will be remembered, was added to it before reautoclaving. To determine if this was the case, D° was reinoculated with *B. paratyphosus alpha,* incubated forty-eight hours, and then compared colorimetrically with D +, the uninoculated sample. No detachable change in color had taken place, showing that the high temperature and pressure incidental to the process of autoclaving did not increase the fermentable sugar content. If hydrolysis had occurred, a slight acidity would have been produced in D° caused by the acid of fermentation of the hexoses, dextrose and galactose.

To show that litmus milk subjected to such treatment can still give the reaction, part D +, to which had been added 0.05 per cent. dextrose, was now inoculated with a loopful of the milk from D°, which, it will be remembered, did not show the color change after forty-eight hours' incubation. After incubating D + for forty-eight hours, the lilac color change had appeared, when it was compared with D°, which remained neutral. The negative result in D°, therefore, is best explained by the absence of a fermentable substance. The temperature incidental to the process of sterilization, therefore, cannot be responsible for the presence of this fermentable substance in litmus milk in this instance.

A quantitative estimation of hexose in milk was made by comparing the degree of lilac color change produced by the fermentation of varying amounts of dextrose in litmus milk, and by titration of the acid produced by fermentation.

In this test it was necessary to use samples of milk from which the fermentable substance had previously been removed through fermentation, and then neutralized to the color of a control sample of sterile neutral litmus milk. These samples were prepared by the method used in preparing the sample D° just described. After they were so prepared, varying amounts of dextrose (0.05, 0.06, up to 0.15 per cent.) were then added to the sample, respectively, and after sterilization each sample was inoculated with *B. paratyphosus alpha.* These samples containing known quantities of dextrose were then incubated twenty-four hours, and it was found that the one containing 0.08 per cent. of dextrose corresponded most closely in color with that observed in normal milk cultures. This method of comparing the degree of color-change, therefore, shows roughly 0.08 per cent. dextrose in milk.

In the method of titrating the acid formed by fermentation a twenty-four-hour normal milk culture and the twenty-four-hour 0.08 per cent. dextrose culture described above were titrated to neutrality, and found to require the same amount of alkali, namely, 9.2 c.c. of 10/n NaOH. This amount of alkali corresponds to 0.083 gm. of lactic acid and to 0.083 gm. of dextrose, according to the equation $C_6H_{12}O_6 = 2\ HC_3H_5O_3$. It cannot be stated positively that this acid is lactic acid, since there is no specific test for this substance which is applicable under these conditions. It can, however, be confidently stated that formic acid is not present in appreciable quantity.

A liter of normal milk was inoculated with *B. paratyphosus alpha* and incubated seventy-two hours. The culture was then concentrated to a volume of

100 c.c. by evaporation and then extracted with ether. The ether extract gave a definite Ufflemann test for lactic acid. This test, however, as stated above, is not conclusive under these conditions. A second liter sample was similarly prepared and incubated. The culture was shaken up with freshly precipitated cupric oxid. After evaporating to 50 c.c. and then acidifying with sulphuric acid in order to convert the copper formate into formic acid, a few cubic centimeters of this sample were distilled and the distillate tested with ammoniacal silver nitrate. No reduction appeared. A control test similarly carried out, using *B. coli,* however, gave a positive test.

These two methods, therefore, agree in showing an amount of fermentable substance in milk corresponding to about 0.08 gm. of dextrose. In other words, in (1) the intensity of the color change in normal litmus milk cultures corresponded to the color change produced by the fermentation of 0.08 gm. of dextrose in 100 c.c. of neutral milk; in (2) the amount of N/10 NaOH required to neutralize the acid formed in a normal litmus milk culture corresponded to 0.083 gm. of dextrose (estimated as lactic acid) in 100 gm. of milk.

Experiments were made to determine if this lilac-color reaction can be obtained in milk which has not been subjected to the high temperature used in the sterilizing process, that is, in milk directly from the cow and untreated in any manner.

Milk that is to be used for cultural purposes must be sterilized; the usual method is to autoclave it at 120 C. for fifteen minutes. The evidence thus far presented for the occurrence of a hexose sugar in milk is based on bacteriological reactions obtained with milk which has been prepared in this manner. It is desirable to make similar tests, substituting absolutely freshly drawn milk for that which has been subjected to a variety of manipulations. If the reactions are as definite in the unchanged milk, they would lend additional evidence to the view that the fermentable substance is not produced in the process of autoclaving — in other words, that it is a normal constituent of milk as it is drawn from the cow. It is necessary to obtain milk samples free from bacterial contamination for this purpose. Bacteria are normally present in the udder, especially in the teats and in the upper parts of the milk ducts. Harding and Wilson[6] have shown, however, that the middle portion of the milking, especially of the front quarters of the udder, shows a very low bacterial count. The milk samples employed in this test were obtained by discarding the fore milk of the right front quarter of the udder and drawing the middle portion through a sterile glass cannula, one end of which was inserted into the teat, the other end passing through the cotton plug of a sterile 250 c.c. flask. This milk was then removed into sterile test tubes from the flask by a sterile pipet in 10 c.c. quantities. Half of the samples thus obtained were then inoculated with *B. paratyphosus alpha* and the entire number of samples placed in the incubator at 37 C. After twenty-five hours all the samples, both inoculated and uninoculated, were colored with litmus. The difference in color of the inoculated and uninoculated tubes was unmistakable; the inoculated samples showed uniformly the typical

6. *Tech. Bull.,* 1913, No. 27, N. Y. Agricultural Experiment Station.

lilac-color change mentioned above, while the uninoculated samples were uniformly neutral. This shows conclusively that the reaction may be obtained in freshly drawn milk.

CHEMICAL EVIDENCE

The literature relating to the chemical composition of milk makes no definite mention of the occurrence of a hexose as a normal constituent of milk, yet the constant production of acid as shown by the bacteriological tests given above seems to warrant the conclusion that this lilac-color reaction is due to the formation of acid by fermentation of a hexose sugar. However, it was considered essential to employ some chemical test for verifying the bacteriological reactions.

In a chemical examination of milk for the presence of this substance, the most obvious difficulty was to obtain the whey of the milk free from proteins and other substances which would obscure the subsequent chemical tests, without at the same time causing hydrolysis of lactose into dextrose and galactose in the process.

Another difficulty is encountered in attempting to demonstrate the presence of a trace of a sugar with six carbon atoms in the presence of a relatively large quantity of a sugar with twelve carbon atoms.

Polariscopic methods are not sufficiently delicate for this purpose, and of the precipitation tests only one merits any consideration. This is Barfoed's test, which has been used frequently for detecting hexoses in the presence of bioses. This test proves to be delicate to one part in about 40,000 under suitable conditions. A modification of this test was finally devised and is here given:

Barfoed's test is made by boiling the material to be tested, a few drops at a time, with 5 c.c. of a solution containing 4.5 gm. of copper acetate and 1.2 c.c. of 50 per cent. acetic acid per 100 c.c. of water. Some observers claim that lactose is slowly hydrolyzed to dextrose and galactose in the presence of this reagent, which obviously would interfere with the demonstration of any hexose already present. It was found, however, that with the modification as here given, hydrolysis manifested itself only after vigorous and continued boiling for a period much longer than was necessary for the entire process.

One hundred c.c. of milk at 37 C. are shaken up with 2 gm. of copper acetate and filtered at once. The resulting clear whey is then titrated with silver nitrate solution to remove the chlorids which interfere with the subsequent precipitation of cuprous oxid in the last step in the process. It should be emphasized that the least excess of silver nitrate is to be avoided, since the lactose as well as any hexose sugar present will cause its reduction, thus obscuring the reaction. On the other hand, if too little silver nitrate is added, the unprecipitated chlorids, even in very small amounts, prevent the precipitation of cuprous oxid as mentioned above. For this reason the filtrate should be tested with silver nitrate, refiltered and again tested until not more than a trace of chlorid can be precipitated from the whey. The chlorid content of different samples of

milk has been found to vary somewhat, and for this reason the removal of the chlorids constitutes the most tedious part of the process. To avoid unnecessary dilution of the whey a 2.5 per cent solution of silver nitrate is used; slightly more than 4 c.c. (usually about 4.3 c.c.) are required for 25 c.c. of the whey.

After filtering out the precipitated chlorids, the whey is then heated to 90 C. and filtered while still hot to remove phosphates. Two parts of this whey when boiled with one part of a solution containing 8 per cent. copper acetate and 1 per cent. glacial acetic acid will show a slight, dark red precipitate of cuprous oxid. The precipitate is so small in amount that the tubes should stand several minutes or even hours; when viewed against a black background by reflected light the precipitate is seen adhering to the sides or settled to the bottom of the tubes. It cannot be seen distinctly by transmitted light.

To determine the ability of various organisms to ferment this reducible substance, the following tests were carried out for each of the following organisms: *B. alkaligenes* and *B. subtilis* — known to be unable to ferment dextrose — and *B. paratyphosus alpha* and *beta, B. typhosus,* and the Flexner and Shiga types of dysentery bacilli — known to ferment dextrose. Flasks containing 100 c.c. of sterile milk were inoculated, respectively, with each of the above-named organisms and incubated forty-eight hours. The whey from each flask was then analyzed for reducing substances by the above method, and its absence demonstrated in the cultures of the dextrose-fermenting ones just named. On the contrary, *B. subtilis* and *B. alkaligenes,* non-dextrose-fermenting bacteria, had not appreciably diminished the original quantity of this reducing substance, as shown by the fact that the cuprous oxid precipitate appeared in the usual quantity. In other words, this reducible substance, whatever it may be, is not appreciably reduced in amount by non-dextrose-fermenting organisms, but it is quantitatively removed by those fermenting dextrose. Furthermore, analyses of 100 c.c. samples of milk, after incubating at 37 C. for periods of eighteen, twenty-four, thirty-six and forty-eight hours, showed that *B. typhosus* and *B. paratyphosus alpha* and *beta* could remove this reducible substance completely in twenty-four hours; while the Flexner and Shiga types of the dysentery bacilli required forty-eight hours' incubation to completely remove it. These observations accord with what is known of the relative vegetative activity of these organisms, and therefore lends additional support to the assumption that this reducible substance is a hexose, fermentable by them.

It might be objected at this point that certain substances may have been present in the material introduced at the time of inoculation, or formed during the growth of the dextrose-fermenting organisms, which may have prevented the precipitation of cuprous oxid, thereby making the test appear to show absence of hexose, as happens in the case when all the chlorids are not removed. This possibility of error was avoided by the use of the following control tests: each of the forty-eight-hour-old cultures of the dextrose-fermenting organisms mentioned above were rendered protein-, chlorid- and phosphate-free by the process just outlined, and in each instance like amounts of whey, now presumably free, by fermentation, from the reducing substance, were placed in two test tubes, A and B. The required amount of the reagent was then added to each tube, and to A was added dextrose in a concentration of 0.01 per cent. After boiling both tubes, A and B, a distinct precipitate was obtained in A and none in B. The negative test in Tube B shows that the organisms have reduced the quantity of reducible substance at least below the concentration recognizable by this test, because Tube A, known to contain 0.01 per cent. dextrose, gives a positive test. This control also shows that lactose is not appreciably hydrolyzed either by the

reagent itself or by the process of freeing the wheys of the proteins, phosphates and chlorids, otherwise Tube B, which contained no addition of dextrose, would give a reduction with the dextrose and galactose so formed. Furthermore, the control enables one to determine if chlorids, which might have inadvertently not been removed, are interfering with the test. In such a case, Tube A, known to contain dextrose, would then fail to give the precipitate.

Having now a method for proving the absence of a hexose in milk after incubating with some dextrose-fermenting organism, the next step in the investigation was to verify by chemical methods whether or not some hexose is produced by the hydrolysis of lactose during the normal process of sterilization.

Two hundred c.c. of sterile milk were inoculated with *B. paratyphosus alpha* and incubated forty-eight hours. To determine if this milk now contained the reducible substance, the sample was divided into two equal parts, A and B. A was analyzed by the above method and shown to be free from this reducible substance. The other portion, B, shown by analysis of A to be free from reducible substance, was then autoclaved in the usual manner for sterilizing milk for cultures, that is, at 120 C. for ten minutes. If sterilization is responsible for the formation of dextrose and galactose in milk, chemical analysis of this latter portion should show the usual cuprous oxid precipitate. The analysis, however, failed to show the presence of a reducing substance, indicating that if hydrolysis of lactose had taken place in the process of autoclaving, the amount of sugar with six carbon atoms so produced was insufficient to give the test.

A number of 10 c.c. samples of normal milk were analyzed by the method given above and the cuprous oxid precipitates weighed in each instance. The average weight of precipitate obtained from 10 c.c. of milk was .003 gm. of cuprous oxid. Other 10 c.c. normal milk samples were then inoculated and incubated in order to free the samples of the reducible substance already present. These samples were then treated with 0.1 per cent. dextrose and analyzed by the above method. The average weights of cuprous oxid precipitate from each of these samples was .005 gm. According to the proportion .003 g.: .005 g.:: x per cent. : 0.1 per cent., the amount of reducing substance in milk in terms of dextrose should be .06 per cent. It will be recalled that the method of quantitative estimation of fermentable substances by titration of the acid of fermentation showed an amount corresponding to .08 per cent. dextrose. Milk directly from the cow would probably show 0.1 per cent. or more, as shown by Theobald Smith's estimates. Dextrose being the most frequently and easily fermented of all the fermentable sugars, would naturally occur in slightly varying amounts in market milk, depending on its past history and the age of the sample. Occasionally a sample of milk may fail to give the lilac-color change with dextrose-fermenting organisms. This is apparently due to the fact that the hexose has been decomposed by the action of the bacteria in the milk prior to the addition of the litmus.

It was shown by bacteriological tests that milk freshly drawn from the cow and untreated in any manner could give the typical lilac-color change after inoculation, incubation and coloring with litmus, showing that the substance to which the color change is attributable is normally

present in milk. If now it can be shown that this substance reduces a reagent reducible by sugars containing six carbon atoms, all the evidence will be in favor of the view that this substance, fermentable by dextrose-fermenting organisms and reducible by a dextrose-reducing reagent, is probably dextrose which has found its way from the circulating blood of the cow.

In this test the milk was drawn from the cow directly into flasks and divided into 100 c.c. quantities. The entire process of the analysis was carried out immediately after milking. On boiling the wheys of these samples with the reagent as before, the usual dark-red cuprous oxid precipitate, apparently in the usual quantity, was obtained, showing that regardless of where and by what mechanism this substance had its origin, a second reducible, fermentable carbohydrate, probably dextrose, occurs as one of the normal constituents of the mammary secretion, in amounts approximating the concentration of dextrose found in the circulating blood of the cow.

CONCLUSION

Bacteriological and chemical evidence has been presented which indicates that milk normally contains a substance which reacts like dextrose.